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Monoclonal Antibody Mapping of the Envelope Glycoprotein of the Dengue 2 Virus, Jamaica

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Although dengue (DEN) virus is the etiologic agent of dengue fever, the most prevalent vector-borne viral disease in the world, precise information on the antigenic structure of the dengue virion is limited. We have prepared a set of murine monoclonal antibodies (MAbs) specific for the envelope (E) glycoprotein of DEN 2 virus and used these antibodies in a comprehensive biological and biochemical analysis to identify 16 epitopes. Following domain nomenclature developed for the related flavivirus, tick-borne encephalitis, three functional domains were identified. Five epitopes associated with domain A were arranged in three spatially independent regions. These A-domain epitopes were destroyed by reduction, and antibodies reactive with these epitopes were able to block virus hemagglutination, neutralize virus infectivity, and block virus-mediated cell membrane fusion. Domain-A epitopes were present on the full-length E glycoprotein, a 45-kDa tryptic peptide representing its first 400 amino acids (aa) and a 22-kDa tryptic peptide representing at least aa 1–120. Four epitopes mapped into domain B, as determined by their partial resistance to reduction and the localization of these epitopes on a 9-kDa tryptic or chymotryptic peptide fragment (aa 300–400). One domain-B-reactive MAb was also capable of binding to a DEN 2 synthetic peptide corresponding to aa 333–351 of the E glycoprotein, confirming the location of this domain. Domain-B epitopes elicited MAbs that were potent neutralizers of virus infectivity and blocked hemagglutination, but they did not block virus-mediated cell-membrane fusion. Domains A and B were spatially associated. As with tick-borne encephalitis virus, determination of domain C was more problematic; however, at least four epitopes had biochemical characteristics consistent with C-domain epitopes.

INTRODUCTION

While dengue continues to be the most important arthropod-borne flaviviral disease, and many immune reagents, including monoclonal antibodies (MAbs) and antipeptide antibodies, have been prepared against these viruses, our understanding of the antigenic structure and intermolecular interactions of the dengue (DEN) virus surface glycoproteins lags behind that of another flavivirus, tick-borne encephalitis (TBE) virus. Studies with TBE virus have led to good understanding of some of the structure/function relationships of the virion structural proteins, especially the major flavivirus envelope (E)-glycoprotein (Mandl *et al.,* 1989; for review see Heinz and Roehrig, 1990; Roehrig, 1997).

Using MAb analysis, researchers have identified three antigenic domains (A, B, and C) on the E-glycoprotein of TBE virus (Guirakhoo *et al.,* 1989). The A domain [approximately amino acids (aa) 50–130 and 185–300] was identified as a linearly discontinuous domain, separated by a C domain (approximately aa 130–185). The A-domain structure is stabilized by five disulfide bridges and, therefore, contains epitopes susceptible to reduction, many of which elicit virus-neutralizing antibodies. Functional A-domain epitopes as measured by MAb binding are found only on the intact E glycoprotein or on a 45-kDa tryptic fragment approximately composed of aa 1–380. Much of the antigenicity of the B domain requires a disulfide bond between Cys 11 and Cys 12, is far more conformationally stable, and is identifiable as a trypsin/chymotrypsin-resistant core of 9 kDa (approximately aa 300–400)(Winkler *et al.,* 1987). Epitopes located in the B domain can elicit virus-neutralizing or hemagglutination-inhibiting MAbs. The TBE C domain is conformationally stable; however, many C-domain epitopes are lost following proteolytic digestion. Only one of six C-domain epitopes elicited a MAb with virus-neutralizing activity.

Recently, the 2 Å molecular structure of the amino-terminal 45-kDa tryptic fragment of the TBE E glycoprotein homodimer was solved (Rey *et al.,* 1995). The E glycoprotein folds into three distinct domains (I, II, and III), which correlate well to the previously defined domains C, A, and B. Because the locations of the E-glycoprotein Cys residues are conserved among all flaviviruses, it is generally assumed that this overall structure is the same for all flaviviruses, including DEN virus. It must be noted, however, that similar studies with DEN virus or other flaviviruses have not been done, so extrapolation of results from TBE virus studies to all other flaviviruses needs to be experimentally confirmed.

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A variety of antigenic mapping experiments have been performed with DEN viruses by using expressed recombinant fragments to further define the epitopes on the DEN virus E glycoprotein (Innis *et al.,* 1989; Aaskov *et al.,* 1989; Mason *et al.,* 1989; Megret *et al.,* 1992; Trirawatanapong *et al.,* 1992; Lin *et al.,* 1994; Hiramatsu *et al.,* 1996). Using a panel of synthetic peptides derived from the deduced amino acid sequence of the E glycoprotein of DEN-2 Jamaica (JAM) virus, which included most of its 495 aa, we determined previously that 11 peptides defined four antigenic regions (aa 1–55; 79–172; 225–249; and 333–388) that could elicit antiviral antibody reactive in an enzyme-linked immunosorbent assay (ELISA), in which virus was absorbed to the ELISA plate (Roehrig *et al.,* 1990, 1994). These regions could be mapped to domains A (aa 1–55; 79–142; 225–249), C (aa 79–172), and B(aa 333–388). Two peptides (aa 35–55 and 352–368) elicited low levels of virus-neutralizing antibody in mice. One observation of particular interest was the ability of some of these antipeptides to react better with virus that had been exposed to low pH. These peptides were localized in two linearly discontinuous regions, aa 58–120 and 225–249. One of these regions contained the putative flavivirus fusion sequence (aa 98–110) that is presumably exposed by treatment of the E glycoprotein with acid. A subsequent study linked mutations in the E glycoprotein aa 6, 134, and 153 with alterations in virus-mediated cell-membrane fusion (Guirakhoo *et al.,* 1993).

A limited MAb map for DEN-2 virus has been developed (Gentry *et al.,* 1982; Henchal *et al.,* 1985; Monath *et al.,* 1986; Jianmin *et al.,* 1995). Seven E-glycoprotein epitopes were identified on the DEN-2, New Guinea C (NGC) strain by Henchal *et al.* (1985). In this study, epitopes arranged themselves in a spatial continuum when analyzed by antibody competitive binding assay (CBA). One MAb that mapped an epitope not in this spatial continuum was shown to be reactive with the prM protein. Results from subsequent studies generally confirmed these results with very few exceptions (Monath *et al.,* 1986; Jianmin *et al.,* 1995). One notable difference was the identified spatial relationship of the epitopes defined by MAbs 3H5 and 4E5. While these studies identified epitopes of the DEN 2 virus E glycoprotein, they did not investigate the structure–function properties and relationships of this protein in the same detail as has been done with the TBE virus. To document the antigenic structure of the DEN virus E glycoprotein, we now report the isolation and characterization of a set of DEN 2 virus-elicited IgG MAbs. We have used these MAbs to map more precisely the biologically important epitopes and domains on the DEN-2 virus E glycoprotein. Not unexpectedly, our results are very similar to those determined for TBE virus. This expanded understanding of the DEN-2 virus E-glycoprotein structure and the availability of these well-characterized anti-DEN MAbs should be most useful in DEN virus vaccine design.

RESULTS

MAb characterization and epitope identification

Ten individual fusions were performed, by using mice immunized with high-pH virus alone, with low-pH virus alone, or with a combination of low-pH virus followed by high-pH virus. Combining the MAbs developed here with the previously characterized MAbs, we identified 16 independent epitopes on the DEN 2 virus E glycoprotein (Table 1). The epitopes were identified by a combination of chemical and serological tests. We were also able to identify one MAb that was specific for the DEN 2 virus nucleocapsid protein, MAb 1A2A-1. This MAb and the previously prepared MAb reactive with the prM protein (MAb, 2H2) served as excellent control antibodies in the various serological assays. Three of our MAbs defined epitopes with serologic activities and chemical stabilities with three Mabs previously described by Henchal *et al.* (1985): 9A3D-8 (similar to 3H5), 1A1D-2 (9D12), and 6B6C-1 (4G2). None of the 16 E-glycoprotein epitopes were more accessible on low-pH-treated virus when compared to high-pH virus when either plate-bound or antibody-captured viruses were used in ELISA (data not shown). The serologic specificity of these 16 MAbs ranged from DEN 2 virus specific to flavivirus group reactive (Table 1). The MAb 4E5 reacted with DEN 1, 2, and 3 viruses in these ELISA assays. This observed 4E5 cross-reactivity was different than that previously reported by Henchal *et al.* (1985), but confirmed later 4E5 cross-reactivity reported by Kaufman *et al.* (1987).

Conformational requirements for epitope activity

To determine the conformational requirements of these epitopes, virus–antibody reactivity was determined by using immunoblotting with and without reduction with 2-mercaptoethanol (Table 1). Three reactivity patterns were identified: partial resistance to 2-mercaptoethanol (1A6A-8, 9A3D-8, 1A1D-2, 10A4D-2, and 10A1D-2); sensitivity to 2-mercaptoethanol (2B3A-1, 1A5D-1, 2H3, 1B4C-2, 4A2B-4, 4E5, 13B7, 1B7 and 6B6C-1); and sensitivity to SDS denaturation (4A5C-8 and 9A4D-1).

Virus neutralization epitopes

MAbs that neutralized infectivity define epitopes that could be either somewhat conformationally stable (9A3D-8, 1A1D-2, and 10A4D-2) or conformationally dependent (4E5, 1B7, and 6B6C-1) (Table 1). Not surprisingly, most MAbs that neutralized virus infectivity (MAbs 9A3D-8, 1A1D-2, 10A4D-2, 1B7, and 6B6C-1) bound well to epitopes that were accessible on the

TABLE 1

Serological, Biological, and Biochemical Characteristics of DEN 2 E Glycoprotein Epitopes Defined by Monoclonal Antibodies

MAb[a]	Ag[b]	Isotype	Immunoblot[c] −ME	Immunoblot[c] +ME	PRNT[d] (>90%)	Capture ELISA (DEN2)	Indirect ELISA reactivity[e] DEN1	DEN2	DEN3	DEN4	SLE	HI[f] Virus	HI[f] SMB	Fusion inhibition (C6/36)
E glycoprotein														
9A4D-1[g]	L/H	IgG2A	O	ND	−	2.9	<3.0	4.4	<3.0	<3.0	<3.0	<1.0	<1.0	<2.0
1A6A-8	High	IgG2A	E	E	−	2.3	<3.0	5.0	<3.0	<3.0	<3.0	<1.0	<1.0	<2.0
4A5C-8[g]	Low	IgG2B	O	ND	−	2.0	<3.0	3.2	<3.0	<3.0	<3.0	<1.0	<1.0	<2.0
2B3A-1	Low	IgG2A	E	O	−	2.0	<3.0	5.3	<3.0	<3.0	<3.0	<1.0	<1.0	<2.0
9A3D-8	L/H	IgG2A	E	E	+	>5.3	<3.0	>5.3	<3.0	<3.0	<3.0	1.9	<1.0	<2.0
1A5D-1	High	IgG2A	E	O	−	3.5	<3.0	>5.3	<3.0	<3.0	<3.0	<1.0	<1.0	<2.0
2H3	SMB	IgG2A,1	E	O	±	3.8	<4.0	4.6	<4.0	<4.0	<3.0	ND	+	<2.0
IB4C-2	High	IgG2A	E	O	−	>5.3	<3.0	>5.3	5.7	<3.0	<3.0	<1.0	<1.0	<2.0
4A2B-4	Low	IgG2b	E	O	−	2.0	<3.0	3.0	<3.0	3.6	<3.0	<1.0	<1.0	<2.0
1A1D-2	High	IgG2A	E	E	+	>5.3	5.1	>5.3	5.4	<3.0	<3.0	1.6	<1.0	<2.0
4E5	SMB	IgG2A,1	E	O	+	2.6	3.8	4.2	3.2	<2.0	<3.0	ND	+	3.0
1OA4D-2	L/H	IgG2A	E	E	+	5.3	3.9	>5.3	3.9	3.0	<3.0	<1.0	<1.0	<2.0
13B7	SMB	IgG2A	E	O	−	2.3	5.2	4.6	4.3	<3.0	<3.0	ND	ND	ND
1OA1D-2	L/H	IgG2A	E	E	−	2.9	3.6	4.4	3.6	3.3	3.6	<1.0	<1.0	<2.0
1B7	SMB	IgG2A	E	O	+	>5.3	4.3	4.9	4.6	4.6	4.5	ND	+	3.2
6B6C-1	SLE	IgG2A	E	O	±	>5.3	4.6	5.2	4.9	4.9	5.1	2.5	2.2	2.6
prM protein														
2H2	SMB	IgG2A	prM	O	−	2.3	4.6	4.6	4.9	4.3	<3.0	ND	−	<2.0
Capsid protein														
1A2A-1	High	IgG2B	C	C	−	2.0	<3.0	4.1	<3.0	<3.0	<3.0	≤10	≤10	<2.0

Note. Fusion, virus-mediated cell-membrane fusion; SLE, St. Louis encephalitis; IgG, immunoglobulin G.

[a] Monoclonal antibodies analyzed.

[b] Antigen used for mouse immunizations: High, normal virus; low, virus treated at pH 5.0; L/H, 1° inoculation low-pH virus, 2° inoculation normal virus; SMB, suckling mouse brain antigen.

[c] Protein specificities: E, envelope glycoprotein; prM, precursor to M protein; C, capsid protein; O, no reactivity detected; ND, not determined.

[d] Plaque reduction neutralization activity at 1:100 dilution of ascitic fluid.

[e] Reciprocal end point ELISA titers, $\log_{10}$.

[f] Reciprocal end point hemagglutination inhibition tiers, $\log_{10}$, using purified virus or SMB antigen. A "+" or "−" denotes previously reported results (Henchal *et al.,* 1985). ND denotes not determined.

[g] These MAbs were also tested by radioimmunoprecipitation assay. The MAb 4A5C-8 precipitated the E glycoprotein in both the presence and the absence of sodium dodecyl sulfate. The MAb 9A4D-1 did not precipitate the E glycoprotein in the radioimmunoprecipitation assay in either the presence or the absence of sodium dodecyl sulfate.

surface of the native virion, as determined in the antigen-capture ELISA. Two MAbs (2H3 and 1A5D-1) had intermediate binding to captured virus and had no (1A5D-1) or low (2H3) neutralizing activity. One MAb, 4E5, could neutralize virus infectivity without recognizing native virus. The reactivity patterns of these MAbs in the indirect or antigen-capture ELISA with another DEN 2 virus, NGC, were identical to that with DEN 2, JAM (data not shown).

HI and blocking DEN virus-mediated cell fusion

Seven MAbs—9A3D-8, 1A1D-2, 3H5, 2H3, 4E5, 1B7, and 6B6C-1—were capable of blocking hemagglutination of red blood cells by either purified DEN 2 virus or DEN virus-infected SMB. The ability of the previously defined MAbs to block HA of SMB DEN antigen is included in Table 1 for comparison (Henchal *et al.,* 1985). Three MAbs—4E5, 1B7, and 6B6C-1—were capable of blocking DEN virus-mediated cell fusion (Table 1).

Spatial arrangement of epitopes based on CBA

CBA analysis was performed to localize epitopes defined by these MAbs. Two of the 16 MAbs (4A2B-4 and 10A1D-2) were not of high enough binding affinity for CBA analysis, and MAb 13B7 was not in sufficient quantity to include in the CBA. MAb 1A6A-8 only had high affinity for completely reduced antigen and was therefore useless in CBA on nonreduced virus. Thirteen MAbs included in the CBA defined three spatially distinct domains (Fig. 1); 10 epitopes clustered into a single spatial domain. Figure 2 compares the chemical and biological characteristics of each epitope within its spatial configuration. From these data, an epitope map was derived, based on the TBE nomenclature (Fig. 3).

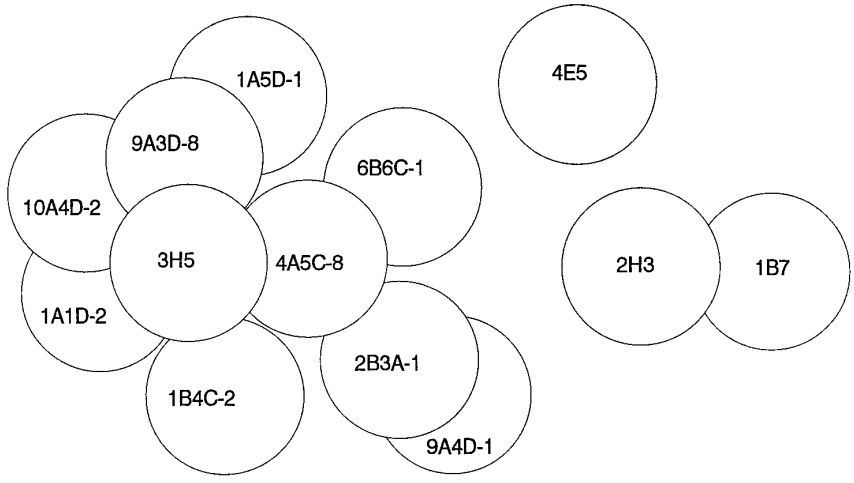

FIG. 1. Competitive binding assay map of DEN 2 E-glycoprotein epitopes. Circles indicate independent epitopes defined by noted MAb. Overlapping circles indicate competition.

Peptide mapping of the E glycoprotein

To further localize epitopes, purified virus was digested with either TPCK–trypsin or α-chymotrypsin. The resultant peptide fragments were separated by SDS–PAGE with or without 2-mercaptoethanol, and reactivity with either MAbs or anti-peptide antibodies was measured by immunoblotting. Only MAb 1A6A-8 reacted with reduced peptide fragments (data not shown). Six tryptic fragments of the E glycoprotein (45, 30, 28 (triplet), 25, 22, and 9 kDa) could be readily identified (Fig. 4A, lane 10) and could be localized based on their reactivity with defined antipeptide antibodies (Figs. 4A and 4B). Two other bands were apparent in these profiles: prM (20 kDa) and capsid (12 kDa). These proteins were not digested under the proteolytic conditions used here. The intact E glycoprotein (50-kDa band) and the 45-kDa fragment reacted with all antipeptides (Fig. 4A, lanes 11 to 16). The 45-kDa fragment most probably corresponds to the 45-kDa TBE tryptic fragment approximately containing aa 1–400 (Fig. 4A, lanes 11 to 16). This was the fragment used to solve the three-dimensional structure of the TBE E glycoprotein.

The 30-, 28-, and 25-kDa fragments were reactive with antipeptides elicited by aa 142–368 (Fig. 4A, lanes 14 to 16), but not antipeptides elicited by aa 35–140 (Fig. 4A, lanes 11 and 13). Based on this antipeptide reactivity pattern, predicted tryptic cleavages sites, and peptide molecular mass, the most likely location of these fragments would be within aa 158–400. The 22-kDa fragment reacted weakly with antipeptides elicited by aa 35–105 (Fig 4A, lanes 11 and 12); therefore, the most likely location of this fragment would be within at least aa 1–120. The 9-kDa fragment that resulted from digestion with either trypsin or chymotrypsin appears to be identical to the 9-kDa tryptic fragment observed with TBE virus. The location of this fragment would be aa 300–400, which is consistent with its reactivity only with the antipeptide 17 (aa 352–368)(Figs. 4A, lane 16, and 4B, lane 3).

Localization of MAb binding site on peptide fragments

Comparing binding of MAbs to either tryptic or chymotryptic peptide fragments revealed at least three binding patterns (Fig. 5). Similar to the CBA results, MAbs 1A1D-2, 10A4D-2, and 9A3D-8 bound the same tryptic fragments: the 45, 30, 28, 25, and 9-kDa peptides (Fig 4A, lanes 1 to 3). These MAbs also bound the same chymotryptic fragments (Fig. 4B, lanes 1 and 2). This binding pattern suggests that the epitopes for these MAbs are located on the 9-kDa fragment which is in turn included within the 45, 30, 28, and 25-kDa peptides (Fig. 5). The second binding pattern was observed for MAbs 10A1D-2 (Figs. 4A, lane 4, and 4B, lane 4) and 6B6C-1 (Fig. 4A, lane 5) and 1B7 (Fig. 4A, lane 6) and was unusual: besides binding to the E glycoprotein and the 45- and 30-kDa fragments, they were the only MAbs to recognize 22-kDa tryptic or chymotryptic fragments (Fig. 5). These MAbs did not bind the 9-kDa fragment, indicating that their defined epitopes were not located within aa 300–400 (Fig. 5). The third binding pattern was represented by Mabs 2H3 and 1B4C-2 (data not shown) which bound only to the E glycoprotein or the large 45-kDa fragment (Figs. 4A, lane 7 for 2H3, and 5).

MAb binding to synthetic peptides

The binding of these MAbs to 25 synthetic peptides, which included most of the 495 aa of the DEN 2 virus E glycoprotein, was analyzed by indirect ELISA (Roehrig *et al.,* 1994). A screening assay was first performed that used the MAbs at 1:1000 dilutions. The binding of

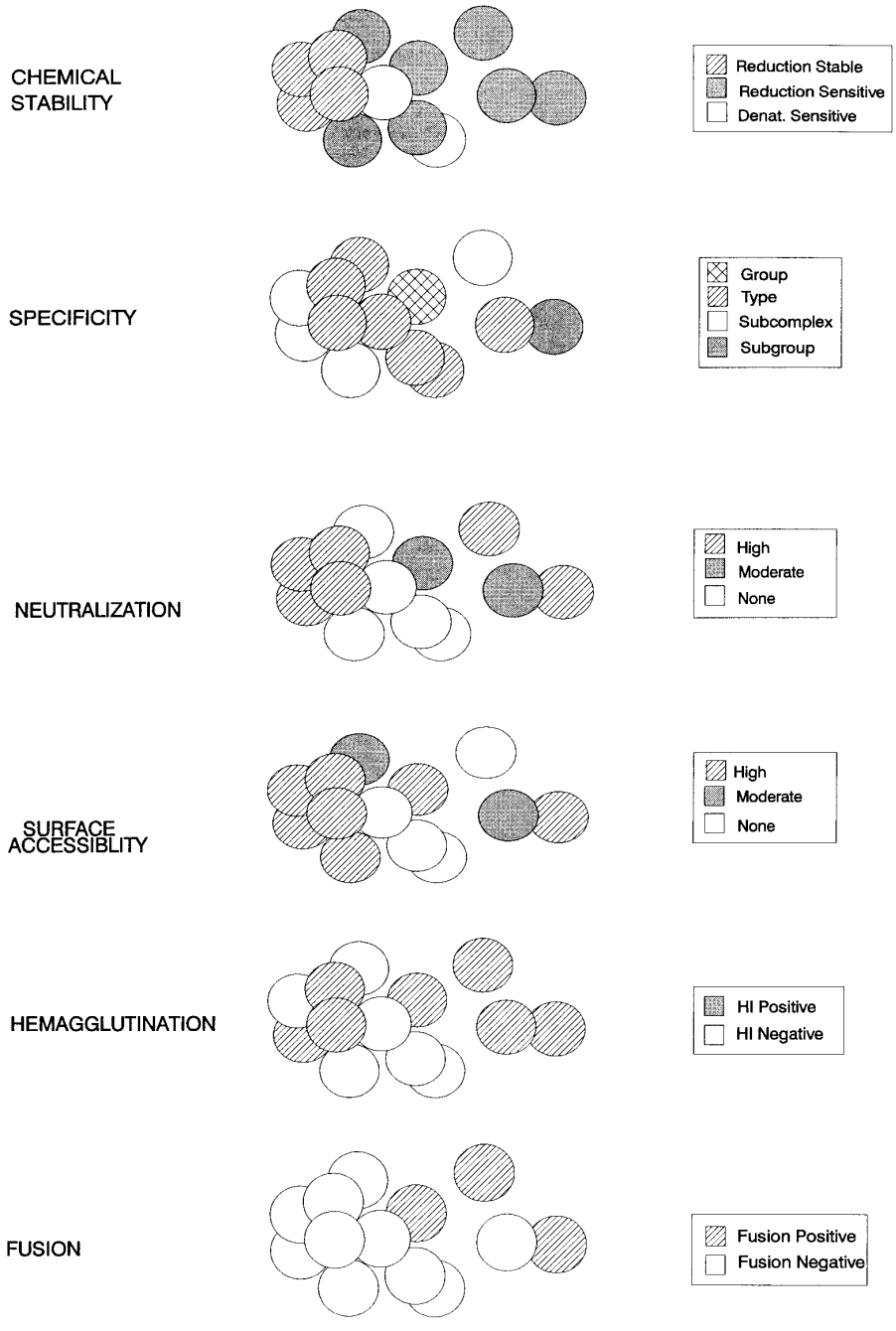

FIG. 2. Summary of the chemical and biological activities of identified E-glycoprotein epitopes as derived from Table 1. For surface accessibility, antigen-capture $\log_{10}$ ELISA titers were interpreted as low (2.0–3.0), medium (3.0–5.0), and high ($\geq$5.0).

each MAb to peptide 470 (aa 470–493, part of the E-glycoprotein hydrophobic tail and not immunogenic) was used as background binding, and absorbance values twofold higher than this were considered positive. Positive MAb reactions in the screening assay were then titrated to end point. Only one MAb, 10A4D-2, demonstrated significant binding to any peptide (16, aa 333–351). The reciprocal $\log_{10}$ end point titer of this peptide binding activity (4.2) was comparable to its virus binding activity (5.3).

DISCUSSION

The results of this study have permitted us to identify and to arrange the DEN 2 E-glycoprotein epitopes in a manner similar to that of TBE virus. The sensitivity to reduction–denaturation of epitopes defined by MAbs 1A5D-1, 6B6C-1, 4E5, 2H3, and 1B7 was consistent with E-glycoprotein A-domain epitopes. Located within domain A is the putative flavivirus membrane fusion sequence (aa 98–110). It is this fusion sequence that is

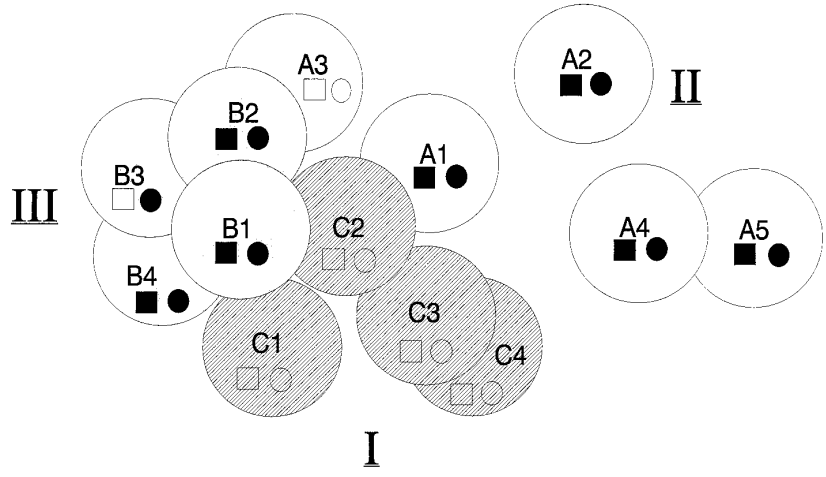

FIG. 3. Epitope assignments of the DEN 2 E glycoprotein. Epitope assignments to domain A (white), domain B (shaded), and domain C (cross-hatched). Squares indicate hemagglutination inhibition activity (filled squares, hemagglutination inhibition positive) and circles indicate neutralization (N) activity (filled circles, N positive). Also listed are domains I, II, and III as defined by X-ray crystallography.

presumed to become more accessible at low pH. Virus-mediated cell-membrane fusion can be blocked by three of these MAbs, 4E5, 1B7, and 6B6C-1. While MAbs 1A5D-1, 6B6C-1, 1B7, and 2H3 readily recognize native virus, the MAb 4E5 can neutralize virus infectivity, but did not bind to intact virus. The only defined mechanism of flavivirus neutralization to date is inhibition of virus-mediated cell-membrane fusion (Gollins and and Porterfield, 1986). It is reasonable to hypothesize that MAb 4E5 effects virus neutralization through this previously defined mechanism and binds to native virus only after it has undergone a low-pH-catalyzed conformational change.

Using antipeptide antibodies, we previously identified two A-domain regions of the E glycoprotein that were made more accessible to antibody binding following virus treatment with low pH. These regions, aa 58–120 (peptides 3-8/1; 3-8/2; and 4-6) and 225–249 (peptide 6), are discontinuous in the primary structure of the E glycoprotein. When the three-dimensional structure of the TBE virus E glycoprotein is analyzed, it can be shown that these discontinuous regions are actually spatially related (Fig. 6). The three-dimensional structure of the TBE glycoprotein homodimer also indicates that the two amino acids associated with virus-mediated cell-membrane fusion (aa 6 and 153, Guirakhoo *et al.,* 1993) and the other area capable of eliciting low-level virus-neutralizing antibody (peptide 35) create a pocket for the putative fusion sequence (aa 98–110) of the alternate monomer (Fig. 6), as was first hypothesized by Rey *et al.,* 1995. It has been suggested that the N-linked carbohydrate acceptor motif at DEN virus aa 153 (aa 154 in TBE

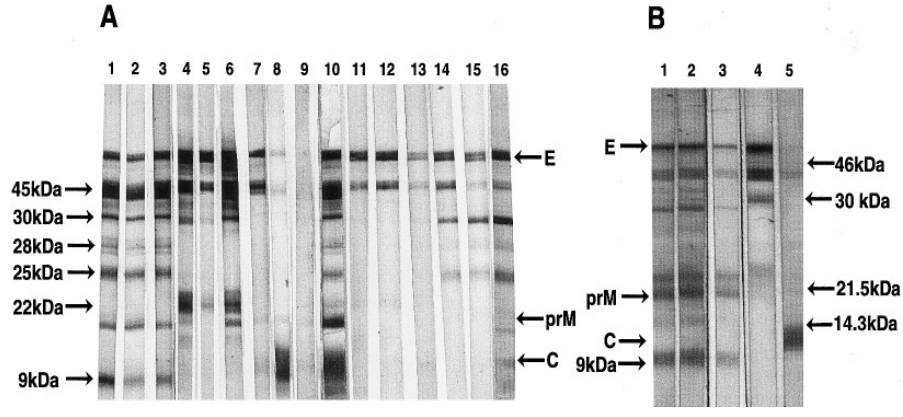

FIG. 4. Antibody reactivities with proteolytic fragments of DEN 2 virus. Immunoblot reactivity of MAbs and antipeptide antibodies with nonreduced tryptic (A) or chymotryptic (B) peptides of DEN 2 virus. Position of molecular mass standards (right of); virion structural proteins (E, prM, C); and peptide fragments are shown. (A) Lanes 1, 1A1D-2; 2, 10A4D-2; 3, 9A3D-8; 4, 10A1D-2; 5, 6B6C-1; 6, 1B7; 7, 2H3; 8, 1A2A-1 (anti-capsid); 9, 1F1 (DEN 1 specific negative control); 10, Gold-blot total protein stain; 11, antipeptide 35 (aa 35–55); 12, antipeptide 4-6 (aa 72–105); 13, antipeptide 4 (aa 121–140); 14, antipeptide 142 (aa 142–172); 15, antipeptide 6 (aa 225–249); 16, antipeptide 17 (aa 352–368). (B) Lanes 1, 9A3D-8; 2, 10A4D-2; 3, antipeptide 17; 4, 10A1D-2; 4, anti-capsid 1A2A-1

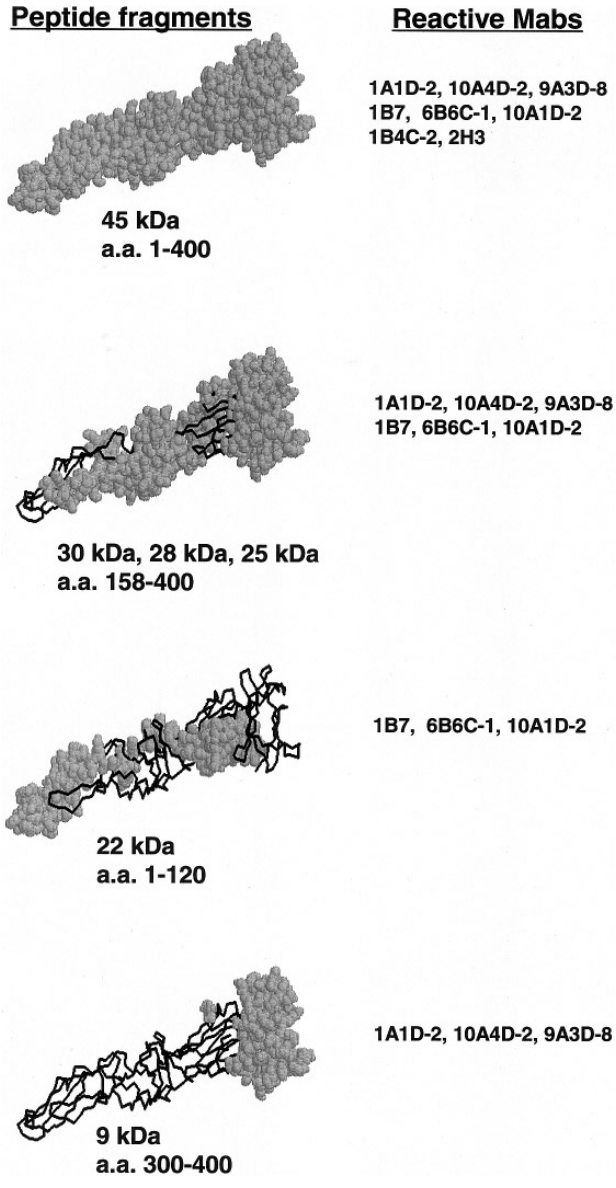

FIG. 5. Localization of MAb reactivity on peptide fragments. Using the defined X-ray diffraction crystal prediction for the tick-borne encephalitis virus E glycoprotein, the location of the observed tryptic peptides (space-filling balls) within the 45-kDa protein backbone (line) is identified. The MAbs reactive with each fragment is listed to the right.

virus) when utilized functions to stabilize the fusion domain and inhibit its release during virion maturation (Rey *et al.,* 1995). The DEN 2 virus aa 153 N-linked glycosylation site is not used, however (Johnson *et al.,* 1994). Instead, the DEN 2 virus glycoprotein is glycosylated at aa 67, which is not spatially related to the fusion domain (Fig. 6). The significance of this change has yet to be defined; however, it calls into question the necessity of glycosylation at aa 153 for proper maintenance of a functional E glycoprotein in DEN 2 virus.

Localization of three of the A-domain epitopes defined by MAbs 6B6C-1, 10A1D-2, and 1B7 was confirmed by the reactivity of these MAbs with the 22-kDa tryptic and chymotryptic peptide (aa 1–120). These MAbs were also somewhat reactive, however, with the 30-kDa tryptic peptide (aa 158–400; Fig. 4A, lanes 4–6). These results seem to indicate that the epitopes defined by MAbs 6B6C-1, 10A1D-2, and 1B7 might be composed of discontinuous parts of the E glycoprotein aa backbone. While this observation seems unusual, a similar result for the epitope defined by MAb 1B7 has been previously reported (Aaskov *et al.,* 1989). Using pepscan analysis, these investigators concluded that the 1B7-defined epitope was composed of aa 50–57 and 127–134 (domain A) and 349–356 (domain B). Investigating the three-dimensional structure of the E glycoprotein reveals that indeed the 22- and 30-kDa peptides are spatially close (Fig. 5). The weak reactivity of antipeptides 35 and 4-6 with the 22-kDa fragment makes unambiguous localization of this fragment difficult. It is clear, however that MAbs and antipeptides recognizing the remainder of the protein fail to bind to this 22-kDa fragment, which indicates that it is not included in a.a. 158–400. It is possible that the secondary structure maintained in the nonreduced gel that is necessary for MAb binding inhibits antipeptide binding which is most efficient with reduced peptides.

The biochemical characteristics of the epitopes defined by MAbs 9A3D-8, 10A4D-2, 3H5, and 1A1D-2 are consistent with location in B domain, based on their partial resistance to reduction–denaturation, their reactivity with the 9-kDa tryptic fragment (aa 300–400), and the binding of MAb 10A4D-2 to peptide 16 (aa 333–351, Fig. 6). These MAbs have potent virus-neutralizing activity. Using a combination of both *Escherichia coli* expression and synthetic peptides, Trirawatanapong *et al.* (1992) mapped the MAb 3H5 binding site to aa 386–397. A peptide derived from this region elicited low-level virus-neutralizing antibody in rabbits (titers of 1:10 to 1:80 at 80% plaque reduction end points). In our hands a very similar peptide (aa 388–400) could not bind to MAb 3H5 nor elicit virus-neutralizing antibody in mice (Roehrig *et al.,* 1990). A more recent study using site-directed mutagenesis of a DEN 2/DEN 4 chimeric virus derived from an infectious cDNA clone identified the E-P-G at aa 383–385 as being critical for MAb 3H5 binding (Hiramatsu *et al.,* 1996). These investigators concluded that aa 383–385 most probably defined the MAb 3H5 binding site. The P-384 residue was clearly the most critical aa in this triad, with viruses mutant at aa 384 losing all reactivity with 3H5 in three different serologic assays. Proline residues introduce β-turns in the polypeptide, so it is still uncertain whether this loss of 3H5 reactivity with aa 384 mutants is the result of the inability of 3H5 to bind aa 384 or the inability of 3H5 to bind a proximal sequence with altered presentation by the substitution of the P at aa 384.

Two other lines of experimental evidence support our definition of the domains of DEN 2 virus. In a previous

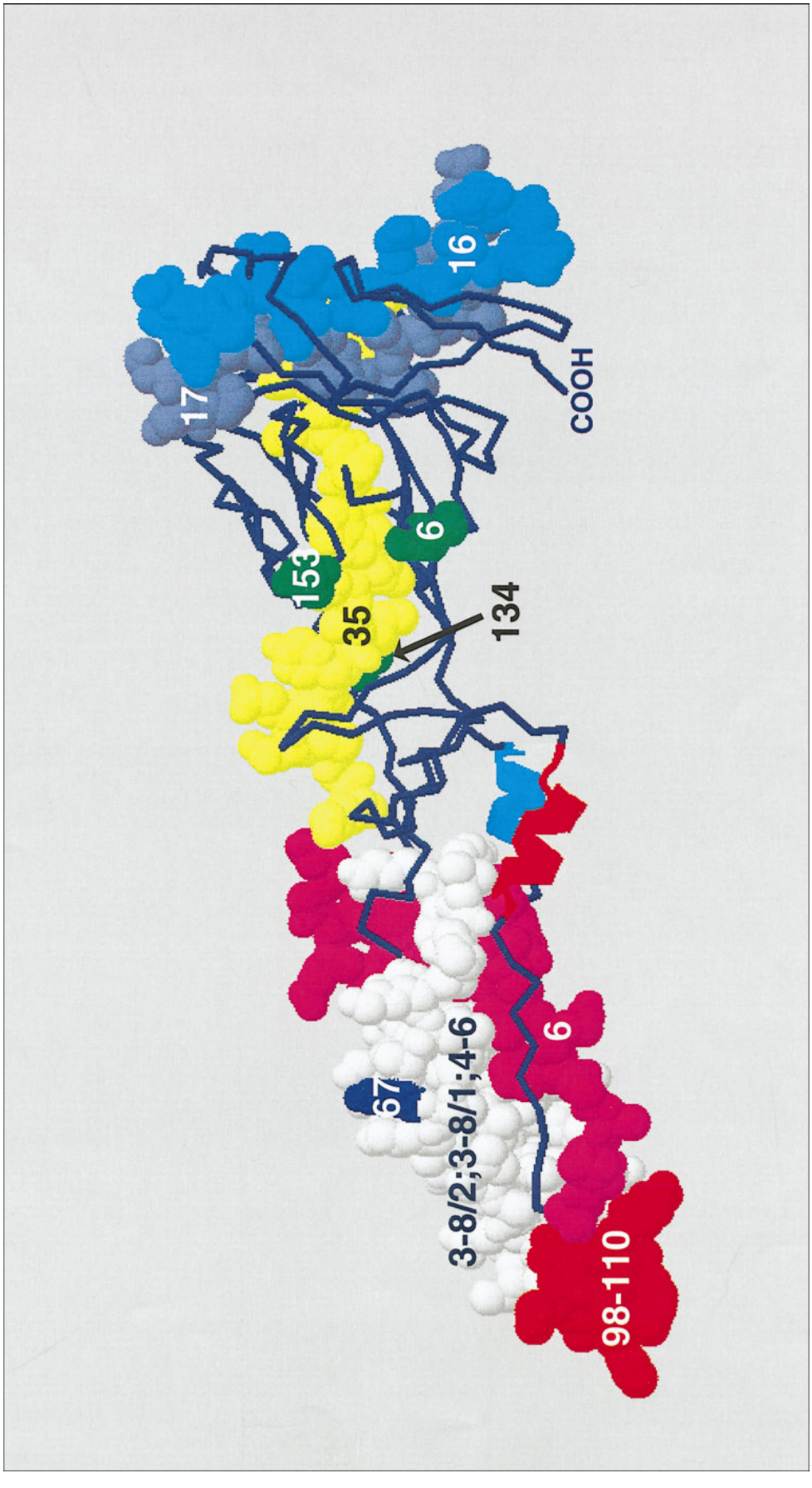

FIG. 6. Location of structurally defined regions of the DEN 2 glycoprotein. X-ray diffraction crystal prediction for tick-borne encephalitis virus E-glycoprotein monomer. Dengue virus regions of interest are presented as space-filled amino acids on a linear blue backbone. Regions are labeled as follows: red, aa 98–110 (putative flavivirus fusion sequence); magenta, peptide 6 (aa 225–249); white, peptides 3-8/2, 3-8/1, and 4-6 (aa 58–121); yellow, peptide 35 (aa 35–55); light blue, peptide 17 (aa 352–368); cyan, peptide 16 (aa 333–351). Location of amino acid mutations in fusion mutants are shown in green: AM (aa 153); FM(aa 6, 134, and 153). Location of the single DEN E-glycoprotein glycosylation site (aa 67) is shown in dark blue. The location of the two E-glycoprotein α-helices, aa 216–223 (cyan) and aa 260–269 (red), are shown to locate the side of the E glycoprotein closest to the membrane.

study, we observed that one of two DEN peptides able to elicit virus-neutralizing antibody was located in the B domain (peptide 17, aa 352–368, Fig. 6). This peptide is adjacent to the binding site of MAb 10A4D-2 (peptide 16, Fig. 6). Our CBA results do not link the epitope defined by 3H5 (B domain) with that of 4E5 (A domain). These results support the epitope map first developed by Henchal *et al.* (1985).

Megret *et al.* (1992) identified six antigenic domains using trp–E-glycoprotein fusion proteins from DEN 2 Jamaica virus. Multiple epitopes were identified by virus-neutralizing MAbs. One B-domain region fragment (aa 298–397) reacted with type-, subcomplex-, complex-, subgroup-, and group-reactive MAbs. Two A-domain fragments (aa 60–135 and 60–205) defined a group-reactive epitope. These domains were also similar to those identified previously by using either pin-bound or free peptides (Aaskov *et al.,* 1989; Roehrig *et al.,* 1990, 1994).

It has been hypothesized that the mechanism of neutralization of B-domain antibodies is blocking attachment of the virus to the cell receptor. The evidence for this is indirect; however, it is based upon the observation that MAb neutralization escape variants, whose mutations are located in the B domain, have altered biological characteristics (Mandl *et al.,* 1989). The virion anti-receptor still remains to be defined. Except for MAb 4E5, all virus-neutralizing MAbs were able to bind to native virus, indicating that these epitopes in both domains A and B were accessible on the virion surface.

Because of the lack of biological activity associated with the C domain (aa 130–185), assignment of this domain is more problematic. The characteristics of four epitopes closely linked in CBA defined by MAbs 4A5C-8, 1B4C-2, 2B3A-1, and 9A4D-1 are most consistent with the C domain. Two of these MAbs (4A5C-8 and 9A4D-1) define the only two epitopes that are susceptible to simple denaturation by anionic detergents. None of these C-domain MAbs has measurable biological activity (HI, N, or blocking fusion). Results with Murray Valley encephalitis virus suggest that the C domain may be the molecular hinge for the acid-catalyzed release of the fusion domain of the E glycoprotein (Guirakhoo *et al.,* 1992).

Because of limitations of antibody avidity or quantity, the location of epitopes defined by four of these MAbs could not be precisely determined. Earlier investigations that used an alternative CBA format localized the epitope defined by MAb 13B7 with other epitopes in the B domain (Henchal *et al.,* 1985). The biochemical characteristics determined here of the epitope defined by MAb 13B7 are more compatible with the A domain. MAb 1A6A-8 defined an epitope that was most reactive on reduced and denatured virus. This was the only epitope with these characteristics and probably explains the inability of MAb 1A6A-8 to react well in the CBA, which uses non-reduced virus. While the binding avidity of either MAbs 4A2B-4 or 10A1D-2 was too low to make them useful in CBA, the biochemical characteristics of the epitope defined by these MAbs makes their location in A domain likely. This conclusion is also supported by the binding pattern of 10A1D-2 with tryptic or chymotryptic fragments, where its binding profile is similar to that of 1B7 and 6B6C-1 (Fig. 4B, lane 4). Because of these limitations, epitopes defined by MAbs 13B7, 1A6A-8, 4A2B-4, and 10A1D-2 could not be included in our CBA map.

The results of this study indicate that while the DEN virus E glycoprotein assumes antigenic characteristics similar to those of TBE virus, subtle differences do occur. This well-defined panel of DEN 2 virus-derived MAbs will allow us to define more precisely the interactions of the DEN virus surface proteins and not require extrapolation of results derived with TBE virus. These MAbs will also be useful in precisely analyzing the antigenic structure of future DEN virus vaccine candidates.

MATERIALS AND METHODS

Virus and cells

The virus used to elicit MAbs in this study was DEN 2, JAM strain 1409. Other strains of DEN viruses used in the ELISA were DEN 1, Hawaii; DEN 2, NGC; DEN 3, H-241; and DEN 4, Dominica. The viruses grown in C6/36 cells and purified on glycerol tartrate gradients (Obijeski *et al.,* 1974) were used as antigens in subsequent serologic assays.

Antibody production and characterization

To assess the effects of low pH treatment of virus on antigenic conformation, male BALB/c mice were immunized for MAb production by using three different inoculation protocols. All inoculations were composed of 50 μg of a particular virus preparation emulsified in Freund's complete adjuvant. Mice were inoculated at days 0 and 16. The first inoculation protocol used purified DEN-2 virus that had been maintained at pH $\geq$7.5 during growth and purification (high-pH virus). The antigen used in the second immunization protocol was high-pH virus that had been denatured by treatment at pH 5.0 (low-pH virus). The final immunization protocol used low-pH virus inoculation followed by high-pH virus inoculation. The protocol for hybridoma production has been previously described (Roehrig *et al.,* 1980). Immunized mice were bled and tested for antiviral antibody in ELISA 20 days after the second inoculation (Roehrig *et al.,* 1990). Antibody-positive mice were boosted one final time, and spleens were harvested 4 days later and used to prepare hybridomas. Hybridomas surviving selection with hypoxanthine–aminopterin–thymidine media were screened by ELISA for reactivity with both high-pH and low-pH viruses. Positive hybridomas were cloned two times in soft agar. High-titered MAbs were prepared as ascitic fluids

from BALB/c mice. MAbs were isotyped by using an ELISA-based isotyping kit for murine antibodies according to the manufacturer's recommendations (Amersham Corp., Arlington Heights, IL.). To make this study as inclusive as possible, anti-DEN virus MAbs that have been previously reported were obtained from Dr. Eric Henchal, United States Army Research Institute of Infectious Diseases. These MAbs—3H5; 2H3; 9D12; 4E5; 13B7; 1B7; 2H2; and 4G2—were prepared from mice immunized with suckling mouse brain (SMB) antigen of DEN 2 virus, NGC strain (Gentry *et al.,* 1982; Henchal *et al.,* 1985). The MAb 6B6C-1 is a flavivirus group-reactive MAb specific for the E glycoprotein, prepared against St. Louis encephalitis virus, and defines an epitope with similar activity as the epitope defined by MAb 4G2 (Roehrig *et al.,* 1983).

Antibody purification

MAbs were purified by high-performance liquid chromatography (HPLC) on a 5-μm, 7.75 $\times$ 100 mm Bakerbond AbxVersa-ten column, on a Beckman System Gold HPLC (Ross *et al.,* 1987). Prior to HPLC, MAb containing ascitic fluids were diluted 1:3 with 25 mM MES [2(*N*-morpholino)ethanesulfonic acid] buffer, pH 6.5 (Buffer A), and were clarified by centrifugation for 30 min at 39,000 rpm in a Sorvall 641 rotor followed by successive filtration through 0.45- and 0.2-μm filters. The column was equilibrated with Buffer A, and 4 ml of clarified ascites was loaded. Antibody was removed with a 0–25% 1 M sodium acetate buffer, pH 7.0, over 30 min at a flow rate of 0.8 ml/min. Protein elution was monitored by absorbance at 280 nm, and 1-ml fractions were collected. Peak fractions were pooled and concentrated into phosphate-buffered saline (PBS) using a Centricon-10 microconcentrator (Amicon, Beverly, MA). Protein concentrations were determined by using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Calif.).

Epitope chemical stability analysis

The protein specificities of the isolated MAbs and the characterizations of the chemical requirement for epitope expression were done by using either reduced or nonreduced immunoblotting, or radioimmunoprecipitation (RIP). The procedures have been published previously. For immunoblotting, purified virus was dissociated in Laemmli sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer with or without 2-mercaptoethanol (Laemmli *et al.,* 1970; Roehrig *et al.,* 1985). After separation of proteins by SDS-PAGE on 12.5% gels, proteins were blotted onto polyvinylidene difluoride-Immobilon membranes (Millipore Corp., Bedford, MA) in a Pharmacia Transblotter (Pharmacia, Uppsala, Sweden). Membranes were blocked with 3% goat serum in PBS overnight and were probed for reactivity by using MAb or polyclonal antibodies. Bound antibodies were detected with goat anti-mouse antibody conjugated to alkaline phosphatase (Jackson ImmunoResearch, West Grove, PA) and blots were developed by using BCIP/NBT substrate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). Total membrane-bound protein was visualized by staining nonblocked membrane with Gold-blot protein stain reagent following the manufacturer's instructions (Integrated Separation Systems, Natick, MA).

MAbs that failed to bind antigen in an immunoblot were further analyzed by RIP by using previously published protocols (Roehrig *et al.,* 1980). Virus used in the RIP assay was radioactively labeled with [^{35}S]methionine (Amersham Corp.). The detergent used in this RIP assay was 1% Triton X-100. Antigen–antibody complexes were precipitated by using goat anti-mouse antibody coupled to Sepharose. Bound viral proteins were visualized by SDS–PAGE on 12.5% gels and autoradiography.

Serological assays

The serological reactivities of these MAbs were tested in plaque reduction neutralization (PRNT) assays, hemagglutination inhibition (HI) assays using either DEN 2 virus SMB antigen or purified DEN 2 high-pH virus, and two forms of ELISA. The first ELISA format was the standard indirect ELISA that used purified high- or low-pH virus adsorbed to Immulon II microtiter plates (Dynatech Industries, Inc., McLean, VA) (Roehrig *et al.,* 1980). The second ELISA format was an antigen-capture ELISA in which DEN 2 virus from tissue culture seed was captured to plates via a polyclonal rabbit anti-DEN 2 virus antibody, which was a gift from Charles H. Calisher. This ELISA format was used to detect epitope expression on the native virion that had not been disrupted by adsorption to polystyrene. After the antigen was coated in this way, the ELISA was completed by using the standard indirect ELISA format. The procedures for PRNT and HI assays have been previously published (Roehrig *et al.,* 1983). The PRNT was performed with a 1:100 dilution of ascitic fluid, and a 90% PRNT cutoff was used to maximize test stringency.

Competitive binding assays

The procedure for antibody CBA mapping of the spatial relationships of epitopes was a modification of our previously published techniques (Roehrig *et al.,* 1982). In this assay, we used biotinylated antibodies in place of enzyme-conjugated antibodies. Purified MAbs were biotinylated by using the Pierce NHS-LC-Biotin Kit, following the manufacturer's recommendations (Pierce Biochemicals, Rockford, IL). The CBA assays were performed with purified virus antigen. Antibody competitors were usually HPLC purified; however, two competing MAbs were used as NH_2SO_4 precipitates (4A2B-4 and 1A1D-2) and four were unpurified ascitic fluids (9A4D-1, 1A6A-8, 4A5C-8,

and 10A4D-2). Both competitor and biotinylated antibodies were added at the same time; therefore, promotion of antibody binding was not observed. Bound biotinylated antibody was detected by the addition of avidin–peroxidase for 1 h at RT followed by 3,3′,5,5′-tetramethylbenzidine substrate (Kirkegaarde & Perry).

Peptide mapping

The procedures for proteolytic cleavage and peptide mapping were similar to those that have been previously published (Guirakhoo *et al.,* 1992). For tryptic digestion, 300 μg of purified DEN 2 virus was digested with 10 μg L-1-*p*-tosylamino-2-phenylethyl chloromethyl ketone (TPCK)–trypsin on ice for 30 min. For chymotryptic digestion, 200 μg of purified DEN 2 virus was digested with 10 μg chymotrypsin at 37°C for 30 min. Digestion optimization assays indicated that the only virion protein digested under these conditions was the E glycoprotein (data not shown). Proteolytic digests were first analyzed by SDS–PAGE on 15% gels and then probed for antibody reactivity by immunoblotting. Most MAb reactivity was lost when peptides were reduced with 2-mercaptoethanol prior to immunoblotting; therefore, only immunoblots of nonreduced peptide mixtures are reported. The preparation and characterization of antipeptide antibodies used to identify the peptide fragment location within the glycoprotein has been published previously (Roehrig *et al.,* 1990, 1994).

Antibody blocking of DEN virus-mediated cell membrane fusion

An assay to measure virus-mediated cell membrane fusion was performed essentially as previously described (Guirakhoo *et al.,* 1992). To effect fusion, DEN 2 virus-infected C6/36 cells were exposed to pH 5.0 at 7 days postinfection. To test the ability of MAbs to block fusion, virus-infected C6/36 cells in 24-well tissue culture plates were pretreated for 1 h at 37°C with dilutions of various MAbs prior to low pH exposure. After MAb treatment, cells were exposed to pH 5.0 as before. Cells were monitored for syncitia formation. Virus-infected, non-MAb-treated cells and uninfected MAb-treated cells were included as controls. The MAb 1H10, which is specific for the E glycoprotein of DEN 4 virus, was included as a negative MAb control.

Molecular modeling of the E glycoprotein

The X-ray crystallographic structure of the E-glycoprotein homodimer of TBE virus was accessed from the Brookhaven protein data base, file 1svb.pdb. Dengue data were input by using the program RasMol, Ver 2.6, available from the RasMol homepage, http://www.umass.edu/microbio/rasmol/, at the University of Massachusetts.

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